EXHIBIT D

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An Anti-CD3 Single-chain Immunotoxin with a Truncated Diphtheria Toxin Avoids Inhibition by Pre-existing Antibodies in Human Blood*

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Diphtheria toxin (DT) is often used in the construction of immunotoxins. One potential problem using DT-based immunotoxins is the pre-existing anti-DT antibodies present in human blood due to vaccination. The present study examined the effect of human serum with pre-existing anti-DT antibodies on the toxicity of UCHT1-CRM9, an immunotoxin directed against CD3 molecules on T-lymphocytes. Sera with detectable anti-DT antibodies at 1:100 or greater dilutions inhibited the immunotoxin toxicity. Experiments with radiolabeled UCHT1-CRM9 indicate that anti-DT antibodies partially block its binding to the cell surface as well as inhibit the translocation from the endosome to the cytosol. The inhibitory effect could be adsorbed using a full-length DT mutant or B-subfragment. A C-terminal truncation mutant could not adsorb the inhibitory effect, suggesting that the last 150 amino acids contain the epitope(s) recognized by the inhibitory antibodies. Therefore, an anti-CD3 single-chain immunotoxin, sFv-DT390, was made with a truncated DT. The IC_{50} of sFv-DT390 was 4.8×10^{-11} M, 1/16 the potency of the divalent UCHT1-CRM9. More importantly, sFv-DT390 toxicity was only slightly affected by the anti-DT antibodies in human sera.

Mutated full-length and truncated diphtheria toxin (DT)[1] molecules are used for making immunotoxins. These immunotoxins show strong cytotoxic effects to their target cells, and some of them have already been used in clinical trials (1–7). Previously, our laboratory constructed an immunotoxin directed against the CD3ε molecule of the T-cell receptor complex, a pan T-cell marker. This construct is made with a monoclonal antibody of mouse-origin, UCHT1, and a binding site mutant of DT, CRM9 (8). The immunotoxin, UCHT1-CRM9, is capable of regressing established xenografted human T-cell (Jurkat) tumors in nude mice (9). A rhesus monkey analog of UCHT1-CRM9, FN18-CRM9, was capable of not only depleting circulating T-cells but also depleting resident T-cells in the lymph nodes.[2] This immunotoxin also delayed skin allograft rejection as compared to antibody treatment and non-treatment controls. FN18-CRM9 has also been used as an adjunct in inducing tolerance to mismatched kidney transplants (24).

In contrast with ricin and *Pseudomonas* exotoxin based immunotoxins, there is a potential problem using UCHT1-CRM9, or other DT-based immunotoxins, in the treatment of human diseases. Most people have been immunized against DT. Therefore these people have a pre-existing anti-DT antibody titer which could potentially inhibit or alter the efficacy of these immunotoxins. This limitation also occurred in our rhesus monkey studies. FN18-CRM9 could deplete T cells in the blood, but to a much lesser extent in animals with anti-DT antibodies, and the T cells repopulated several days earlier compared to those monkeys without anti-DT titers.[2] In order to overcome this antibody mediated inhibition, we undertook the first examination of the effect and the mechanism of human sera containing anti-DT antibodies on UCHT1-CRM9 toxicity. A DT point mutant, a truncation mutant, and DT subfragments were used in an attempt to neutralize the anti-DT effect in human sera. Based on the neutralization data, a single-chain immunotoxin was constructed with a C-terminal deletion mutant of DT which could potentially bypass the inhibitory effect of the pre-existing anti-DT antibodies.

MATERIALS AND METHODS

Cells—Jurkat cells (ATCC) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 25 mM sodium bicarbonate, and 50 μg/ml gentamycin sulfate.

Serum and Adsorbing Molecules—Goat anti-DT serum was provided by Dr. Randall K. Holmes (Uniformed Services University of Health Sciences, Bethesda, MD). Human serum samples were provided by Dr. Henry McFarland (NINDS, NIH, Bethesda, MD). CRM197, an A-subfragment mutant (Gly-52 to Glu) of DT (see Fig. 1*A*), with no enzymatic activity (10) was supplied by Dr. Reno Rappuoli (Biocine-IRIS, Siena, Italy). MSPΔ5, a truncation mutant (amino acid 385) of DT with an additional 5 amino acids at the C terminus was provided by Dr. Richard Youle (NINDS, NIH, Bethesda, MD). Purification of the DT B-subfragment has been described (11).

Immunotoxins—UCHT1-CRM9 synthesis has been described (12). The recombinant immunotoxin, sFv-DT390, was generated in two phases. First the coding sequences for the variable light (V_L) and variable heavy (V_H) chain regions of the UCHT1 antibody were amplified by a two-step protocol of reverse transcriptase-polymerase chain reaction using primers based on the published sequence (13). The 5′ V_L primer added a unique *Nco*I restriction enzyme site while the 3′ V_H primer added a termination codon at the J to constant region junction and an *Eco*RI site. The V_L region was joined to the V_H region by single-stranded overlap extension and the two regions are separated by a $(Gly_3Ser)_4$ linker that should allow for proper folding of the individual variable domains to form a function antibody binding site (14). Second, genomic DNA was isolated from a strain of *Corynebacterium diphtheriae* producing the DT mutant CRM9 (C7[$\beta^{tox-201\,tox-9h+}$]) as described (15). This DNA was used for polymerase chain reaction. The 5′ primer was specific for the toxin gene beginning at the signal sequence and added a unique *Nde*I restriction site. The 3′ primer was specific for the DT sequence terminating at amino acid 390 and added an *Nco*I site in-frame with the coding sequence. The polymerase chain reaction products were digested with the appropriate restriction enzymes and cloned into the *Escherichia coli* expression plasmid pET-17b (Novagen,

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[1] The abbreviations used are: DT, diphtheria toxin; ELISA, enzyme-linked immunosorbent assay; MES, 4-morpholineethanesulfonic acid.

[2] D. M. Neville, Jr., J. Scharff, K. Rigaut, H. Hu, J. Shiloach, W. Slingerland, and M. Jonker, submitted for publication.

TABLE I
Human serum with anti-DT antibodies inhibits the toxicity of UCHT1-CRM9 and the inhibition correlates with the anti-DT titer

Sample	ELISA[a]		Protein synthesis[b]		
	OD ($\bar{X}$ ± S.D.)	Titer	1:10	1:100	1:1,000
				% control	
10010	0.738 ± 0.017	1:750	97 ± 3	79 ± 8	2 ± 0
10011	0.568 ± 0.048	1:500	104 ± 2	13 ± 2	2 ± 0
10012	0.491 ± 0.025	ND[c]	96 ± 3	19 ± 2	2 ± 0
10013	0.411 ± 0.052	1:500	105 ± 8	7 ± 1	2 ± 0
10014	0.390 ± 0.047	1:500	96 ± 2	7 ± 0	2 ± 0
10015	0.353 ± 0.008	1:250	125 ± 6	6 ± 4	2 ± 0
10019	0.359 ± 0.019	1:250	101 ± 7	6 ± 1	2 ± 0
10016	0.141 ± 0.015	1:100	22 ± 1	3 ± 0	2 ± 0
10017	0.100 ± 0.006	<1:100	4 ± 0	3 ± 0	2 ± 0
10018	0.071 ± 0.001	<1:100	2 ± 0	2 ± 0	2 ± 0
Goat	1.450 ± 0.013	$1:10^5$		102 ± 19	104 ± 3

[a] ELISA was performed in triplicate for each serum sample as described under "Materials and Methods." The OD values were derived from 1:100 dilutions and presented as a mean value ± SD. The background value was 0.060 ± 0.02. Titers are recorded as the highest serum dilution that showed a positive reaction in ELISA.

[b] UCHT1-CRM9 (2×10^{-10}) was incubated with different dilutions of serum for 30 min. The mixture was then added to cells as described under "Materials and Methods." Four replicates were performed for each sample. Data are presented as a mean value ± S.D. in percentage of the control counts. UCHT1-CRM9 inhibited protein synthesis to 2.0% of controls. The goat anti-DT serum could be diluted to 1:10,000 and still completely inhibited the toxicity of UCHT1-CRM9.

[c] ND, not done.

Inc., Madison, WI) which had been linearized with *NdeI* and *Eco*RI. The resulting plasmid was used to transform *E. coli* BL21/DE3 cells. Cells were grown to an OD_{590} of 0.5, induced with 0.5 M isopropyl-1-thio-β-D-galactopyranoside (Invitrogen, San Diego, CA) and incubated for an additional 3 h. The sFv-DT390 protein was isolated in the soluble fraction after cells were broken with a French Press and the lysate subjected to centrifugation at 35,000 × *g*.

Protein Synthesis Inhibition Assay—Inhibition assays were performed as described (12) with the following modifications. Immunotoxins were incubated for 30 min with the indicated serum sample or leucine-free medium at room temperature prior to addition to cells. In some experiments the serum was preincubated for 30 min with an adsorbing molecule at the given concentrations to bind the antibodies. The immunotoxin/serum mixture was incubated with Jurkat cells (5×10^4 cells/well in a 96-well plate) for 20 h. A 1-h pulse of [^{3}H]leucine (4.5 μCi/ml) was given before cells were collected onto filters with a Skatron harvester. Samples were counted in a Beckman scintillation counter. Each experiment was performed in 4 replicates. Results were calculated into a mean value, and recorded as a percentage of control cells.

Serum Antibody Detection—Anti-DT antibodies were detected in human serum by ELISA. CRM9 (10 μg/ml) was adsorbed to Costar 96-well EIA/RIA flat bottom plates (Costar, Cambridge, MA) for 2 h and then washed in phosphate-buffered saline containing 0.1% Tween 20. Each well was then incubated with phosphate-buffered saline containing 3% gelatin to prevent nonspecific binding of antibodies to the plastic. Serum samples were diluted in phosphate-buffered saline containing 0.1% Tween 20 and 0.3% gelatin prior to addition to the plate. After a 1-h incubation, the wells were washed as above, and incubated for an additional hour with protein A/G-alkaline phosphatase (1:5,000; Pierce). Wells were washed and phosphatase substrate (Pierce) was added following the manufacturer's directions. After 30 min, color development was stopped with NaOH and the optical density (OD) was measured with a kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA). Each sample was performed in triplicate. Results are presented as OD values and antibody titers.

Endocytosis Assay—UCHT1-CRM9 was iodinated using the Bolton-Hunter reagent (Dupont NEN) as described (16). Jurkat cells were washed twice with binding medium (RPMI 1640 supplemented with 0.2% bovine serum albumin, 10 mM Hepes (pH 7.4) and without sodium bicarbonate). Cells (1.5×10^6) were incubated for 2 h on ice with ^{125}I-UCHT1-CRM9 (1×10^{-9} M) that had been preincubated with serum or binding medium. Unbound antibody was removed by washing the cells twice in phosphate-buffered saline (pH 7.4) with centrifugation and resuspension. Duplicate samples were incubated for 30 min on ice or at 37 °C. One sample from each temperature point was centrifuged at 800 × *g* to separate the total cell associated (pellet) from the exocytosed or dissociated counts (supernatant). Both fractions were counted in a Beckman γ-counter. To determine the amount of internalized immunotoxin, cells from the second sample at each temperature were incubated in low pH medium (binding medium containing 10 mM MES, all of which was titrated to pH 2.0 with HCl) for 5 min to dissociate the surface-bound ^{125}I-immunotoxin (17). Samples were centrifuged at 800 × *g* to separate the internalized (pellet) from the membrane bound (supernatant). Both fractions were counted in a Beckman γ-counter (Beckman).

RESULTS

Serum with Anti-DT Antibodies Inhibits UCHT1-CRM9 Toxicity—Since humans are immunized against DT, the presence of anti-DT antibodies in the serum was determined by ELISA (Table I). In a limited sample population, 80% of the serum samples had an anti-DT antibody titer of 1:100 or above. The vaccination status of the donors was not available. To determine the effect of these antibodies on UCHT1-CRM9 toxicity, the immunotoxin was preincubated with different concentrations of serum and the toxicity of the mixture was assayed (Table I). Serum samples without a significant ELISA OD (2-fold above background) were incapable of affecting UCHT1-CRM9 toxicity at high concentrations of serum (1:10). However, serum samples with a positive ELISA result could neutralize the cytotoxic effect at 1:10 dilution, and those with a high ELISA OD (7–11-fold above background) inhibited toxicity even at a 1:100 dilution. Similar results were seen in assays conducted with monkey serum samples (data not shown).

Sera Do Not Inhibit Endocytosis of UCHT1-CRM9—The inhibitory effect of serum on UCHT1-CRM9 toxicity could be due to prevention of the immunotoxin binding to the cell surface or the endocytosis of UCHT1-CRM9 into the cell. Endocytosis assays were conducted using ^{125}I-UCHT1-CRM9 to determine if either of these processes were affected by anti-DT antibodies present in sera. The results indicate that the presence of serum (goat anti-DT or human) reduces as much as 80% of the immunotoxin counts binding to the cell surface (Table II). While this is a significant reduction in binding, limiting 90% of input immunotoxin (one log less UCHT1-CRM9) in toxicity assays reduces protein synthesis to <25% of controls (data not shown, also see Fig. 2*A*). In contrast, the inhibitory effect of serum containing anti-DT antibodies is 100%. Therefore the effect of the anti-DT antibodies is not all at the level of inhibition of binding to the cell surface. The preincubation of ^{125}I-UCHT1-CRM9 for 2 h on ice and subsequent washing at room temperature resulted in 18–25% of the total cell associated counts internalized (Table II). After incubation for 30 min at 37 °C, there is a doubling of internalized counts both with and without serum, indicating that the same percentage of labeled immunotoxin is endocytosed. The identical dilutions of serum were incubated with non-labeled UCHT1-CRM9 and used in protein synthesis inhibition assays. The results demonstrate that the

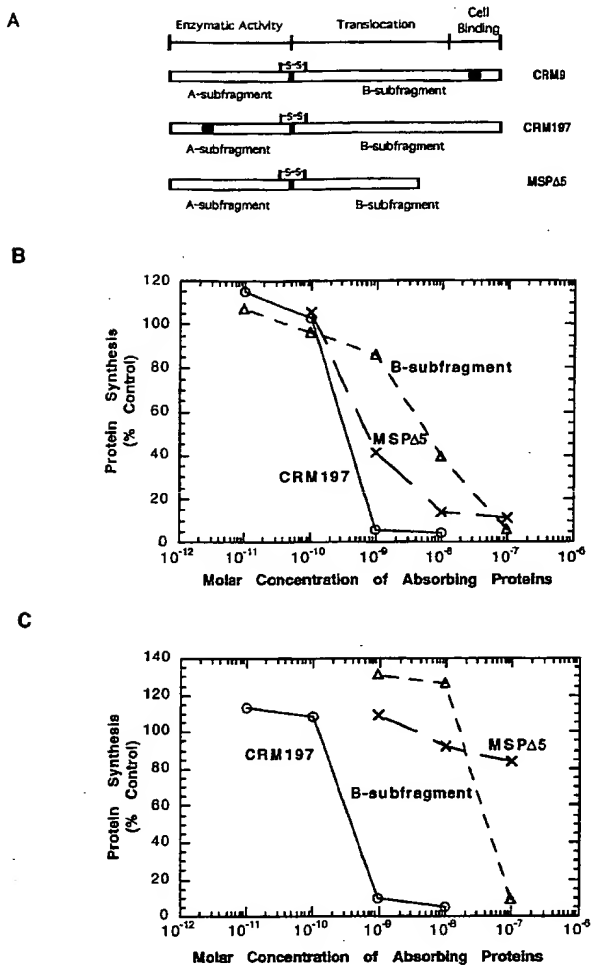

FIG. 1. **The epitopes involved in human serum's inhibition of toxicity lie in the last 150 amino acids of DT.** A schematic diagram of the DT mutants CRM9, CRM197, and MSPΔ5 is presented (*A*). The A- and B-subfragments and their relative size and position are shown. The *filled circle* represents a point mutation as described in the text. Goat (*B*) or human (*C*) serum (human serum was a pool from all samples with positive ELISA for anti-DT antibodies) was incubated with increasing molar concentrations of CRM197 (○), MSPΔ5 (×), or the B-subfragment (Δ) of DT for 30 min at room temperature. To this reaction, UCHT1-CRM9 was added to a final concentration of 1×10^{-10} M. This mixture was then diluted 10-fold onto Jurkat cells in a protein synthesis inhibition assay as described under "Materials and Methods." Immunotoxin incubated with medium only inhibited protein synthesis to 4% of controls. The results are representative of two independent assays.

ratio of immunotoxin to serum used was capable of completely inhibiting the toxicity (Table II), although the endocytosis of UCHT1-CRM9 was not affected.

The Inhibitory Effect of Anti-DT Antibodies Can be Removed by Adsorption—To prevent the inhibitory effect of serum as well as gain insight into the mechanism by which serum inhibits its toxicity, experiments were designed to adsorb the protective anti-DT antibodies from the serum. The serum (a pool of all human sera with positive anti-DT ELISA or goat anti-DT) was preincubated for 30 min with increasing concentrations of CRM197 (an A-chain mutant of DT with no enzymatic activity), MSPΔ5 (a truncation mutant missing the last 150 amino acids), and the purified A- and B-subfragments of DT (Fig. 1*A*). The adsorbed serum was then incubated with UCHT1-CRM9 in protein synthesis inhibition assays. CRM197, the full-length DT-like construct, was capable of completely adsorbing the protective antibodies from both goat (Fig. 1*B*) and pooled human serum (Fig. 1*C*). The B-subfragment of DT is also capable of complete adsorption, however, ~100-fold more is required. The A-subfragment of DT had little or no effect on either serum, although the serum samples were demonstrated to contain antibodies reactive to both the A- and the B-subfragments by Western blot analysis (data not shown). Of interest were the results seen with MSPΔ5, the truncation mutant. Adsorption of goat serum with MSPΔ5 gave a dose dependent removal of the serum's protecting effect (Fig. 1*B*). However, this adsorption could not bring toxicity down to levels obtained when CRM197 or the B-subfragment was used. In contrast to the results observed with the goat serum, MSPΔ5 had little effect on pooled human serum (Fig. 1*C*). These results suggest that the pre-existing anti-DT antibodies important for the protecting effect in human serum are mainly directed against the last 150 amino acids of DT.

TABLE II

Inhibition of UCHT1-CRM9 toxicity by serum does not correlate with inhibition of endocytosis

^{125}I-UCHT1-CRM9 (2×10^{-9} M) was incubated with medium or anti-DT serum (1:4 dilution of human sample 10010 or a 1:1,000 dilution of goat serum; Table I) for 30 min at room temperature. This mixture was added to Jurkat cells (1.5×10^6) for 2 h on ice (final concentration of ^{125}I-UCHT1-CRM9 was 1×10^{-10}). The cells were then washed and endocytosis assays performed as described under "Materials and Methods." The % Bound value represents the cell associated counts divided by the cell associated counts without serum. Non-labeled UCHT1-CRM9 was incubated with the above dilutions of sera and the resulting mixture was used in protein synthesis inhibition assays. The results shown are representative of two independent assays.

Serum sample	Time (37 °C)	Bound	% of bound internalized	Protein synthesis
	min	%		*% control*
	0	100	23.6	ND[a]
	30	100	58.8	3 ± 1
Human	0	20	18.1	ND
Human	30	19	35.9	105 ± 5
	0	100	25.3	ND
	30	100	54.0	3 ± 1
Goat	0	37	24.4	ND
Goat	30	33	50.7	92 ± 14

[a] ND, not done.

sFv-DT390 Is Not Inhibited by Anti-DT Antibodies Present in Human Sera—Having observed that the epitope(s) recognized by the antibodies important for protection lay in the C-terminal 150 amino acids, a single-chain immunotoxin was generated with the first 390 amino acids (out of 535) of DT. Position 390 was chosen for 2 reasons: first, the three-dimensional structure of DT suggested that this position was an external point on the molecule away from the enzymatic domain (18), and second, fusion toxins have been generated with longer DT subfragments with no reports of serum effects (19). The DNA encoding the first 390 amino acids of DT was ligated to DNA encoding the anti-CD3εsFv (V_L linked to V_H using a $(Gly_3Ser)_4$ linker sequence). The predicted molecular mass for the fusion protein is 71,000 daltons and has been confirmed by Western blot analysis of both *in vitro* transcribed and translated protein as well as protein isolated from *E. coli* using goat anti-DT antibodies (data not shown). The toxicity of sFv-DT390 protein, isolated from *E. coli* strain BL21/DE3, was compared to UCHT1-CRM9 in protein synthesis inhibition assays (Fig. 2*A*). The IC_{50} (concentration required to inhibit protein synthesis to 50% of controls) of sFv-DT390 was 4.8×10^{-11} M compared to 2.9×10^{-12} M for UCHT1-CRM9, a 16-fold difference. To demonstrate the specificity of the sFv-DT390 construct, competition experiments were performed using increasing concentrations of UCHT1 antibody as competitor (Fig. 2*B*). The results showed that approximately one-eighth antibody is needed to compete the sFv-DT390 toxicity to 50% as compared to

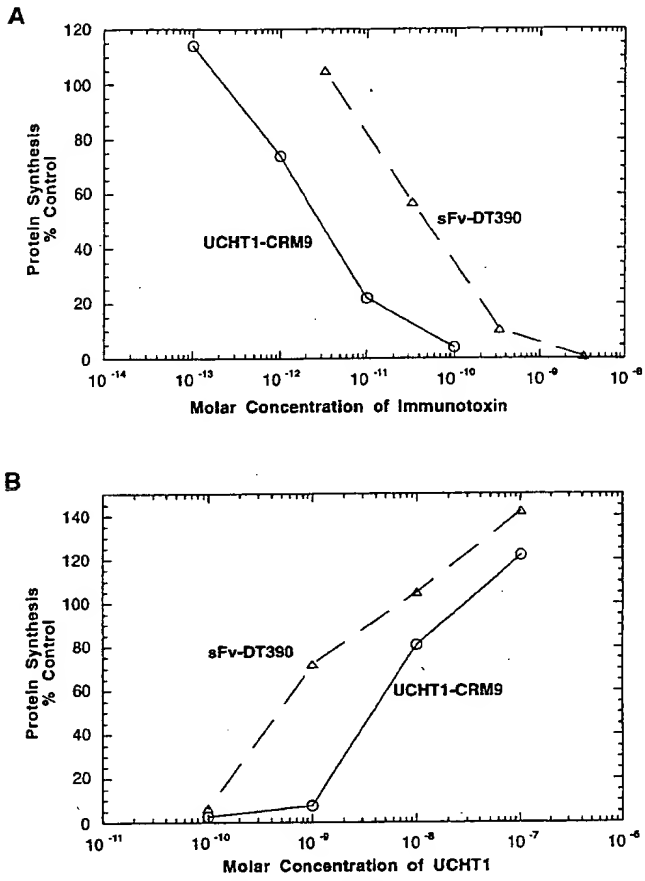

FIG. 2. **sFv-DT390 maintains specificity for the CD3 complex but is 16-fold less toxic than UCHT1-CRM9 to Jurkat cells.** *A*, increasing concentrations of sFv-DT390 (Δ) or UCHT1-CRM9 (O) were tested in protein synthesis inhibition assays as described under "Materials and Methods." The results are an average of four separate experiments. *B*, increasing concentrations of UCHT1 antibody were mixed with a 1×10^{-10} M UCHT1-CRM9 (O) or 3.3×10^{-10} M sFv-DT390 (Δ) and then added to cells for a protein synthesis inhibition assay.

UCHT1-CRM9. The antibody was capable of totally competing toxicity of both constructs thereby showing their specificity. The immunotoxins were then subjected to protein synthesis assays in the presence of increasing dilutions of serum (Table III). UCHT1-CRM9 toxicity was completely inhibited with a 1:10 dilution of the human sera but at a 1:100 dilution toxicity was equivalent to controls without serum. In contrast, the sFv-DT390 immunotoxin is only partially inhibited with the 1:10 dilution of the human sera and the 1:100 dilution no effect on the toxicity. Both immunotoxins are completely inhibited by goat anti-DT serum (1:1,000 dilution). These results indicate that the sFv-DT390 immunotoxin partially evades the pre-existing anti-DT antibodies present in most human sera.

DISCUSSION

The current investigation is the first analysis on the effect of pre-existing anti-DT antibodies on DT-based immunotoxins and to determine the mechanism of the observed effect. Our results indicate that the pre-existing anti-DT antibodies present in human serum inhibit the toxicity of the immunotoxin UCHT1-CRM9. This inhibition of toxicity was also observed with goat anti-DT serum, however, less goat serum was needed to completely inhibit toxicity. The experiments were designed in such a way to mimic the *in vivo* situation. The peak concentration of circulating immunotoxin currently being tested in animal models is 1×10^{-9} M. The immunotoxin concentration incubated with the 1:10 dilution of human serum was 1×10^{-10} M, thus approximating *in vivo* conditions. The inhibition of toxicity correlates with the serum antibody levels as determined by ELISA (Table I), indicating that sera with higher anti-DT titers have a stronger inhibitory effect. Similarly, the goat anti-DT serum which gave the highest ELISA value could be diluted 10,000 times and still completely inhibited UCHT1-CRM9 toxicity. Since this correlation exists, there is no indication that any other component of the serum inhibits the toxicity of UCHT1-CRM9. Furthermore, our data show that a titer of 1:100 dilution is necessary for an inhibition of the immunotoxin toxicity. This is in agreement with data from a clinical trial (20). A construct in which the first 486 amino acids of DT were fused to interleukin-2, $DAB_{486}IL\text{-}2$, was used in lymphoid malignancy patients. A partial response to $DAB_{486}IL\text{-}2$ was observed in several patients who had a anti-DT titer below 1:100 dilution prior to the treatment.

Intoxication of cells by immunotoxins can be subdivided into four general stages: 1) specific binding to the cell surface, 2) endocytosis into the cell, 3) translocation of enzymatic domain of the toxin out of the endosome, and 4) enzymatic inactivation of the target molecule. The results presented indicate that, while the amount of immunotoxin reaching the cell surface is lower in the presence of serum, the same percentage of bound immunotoxin is endocytosed. Taking into account the reduced amount of immunotoxin bound to the cell, the amount of endocytosed immunotoxin should intoxicate the cells to below 25% of controls. However, the immunotoxin had no effect on protein synthesis in the presence of serum containing anti-DT antibodies. Since the A-subfragment of DT could not adsorb the protective effect of serum while the B-subfragment could, the effect of serum is not likely to be at the level of inhibiting enzymatic activity of the toxin. Therefore, it suggests that the anti-DT antibodies affect the translocation of the A-subfragment into the cytosol.

CRM197, B-subfragment, and MSPΔ5 could adsorb the protecting anti-DT antibodies from the goat and rhesus monkey (data not shown) sera. However, among the 3 DT mutants, MSPΔ5 could not prevent the UCHT1-CRM9 toxicity in the presence of the human sera, showing a difference in the anti-DT antibody repertoire among humans, goat, and rhesus monkeys. This difference does not seem to be due to immunization routes, because monkeys used in the present study were not immunized for DT and presumably acquire the antibodies after a natural infection with toxigenic strains of *C. diphtheriae*. Although there were reports showing that rhesus monkeys and humans shared a similar antibody repertoire (21), our results suggest that one must analyze the effect of antibodies from the host for whom immunotoxin treatment is intended.

To overcome the blocking effect of the pre-existing anti-DT antibodies in human sera, there are basically two pathways existing. One is to neutralize the antibodies with non-toxic DT mutants,[3] and the other is to modify the DT structure used for making immunotoxin (3). The antibody neutralization pathway has been tested in our monkey studies of FN18-CRM9 treatment. A 100-fold higher amount of CRM197 was injected 5 min before FN18-CRM9 to adsorb the pre-existing antibodies in 2 monkeys who had an anti-DT titer at 1:1,000 dilution. In one monkey the T cell depletion was as good as in monkeys without anti-DT titers. The other monkey died due to multiple kidney infarcts. It is possible that this condition resulted from immune complex disease precipitated by the neutralization procedure. Thus, serum neutralization may be a potentially dangerous process. Our results showed that although antibodies against

[3] D. M. Neville, Jr. and M. Jonker, unpublished observations.

TABLE III

Anti-DT antibodies present in human sera have reduced effect on sFv-DT390 toxicity

UCHT1-CRM9 or sFv-DT390 (2×10^{-9} M) was incubated with the indicated dilutions of serum for 30 min. The mixture was then added to cells as described under "Materials and Methods." The final concentration of immunotoxin on cells was 1×10^{-10} M. Four replicates were performed for each sample. Data are presented as a mean value ± S.D. in percentage of the control counts. UCHT1-CRM9 inhibited protein synthesis to 5% of controls while the sFv-DT390 inhibited protein synthesis to 18% of controls. The ELISA value was determined using a 1:100 dilution of serum. The results are representative of two independent experiments.

Serum sample	ELISA value ($\bar{x}$ ± S.D.)	Protein synthesis					
		UCHT1-CRM9			sFv-DT390		
		1:10	1:10^2	1:10^3	1:10	1:10^2	1:10^3
		% control					
10012	0.491 ± 0.025 119 ± 24		8 ± 2	ND[a]	47 ± 9	21 ± 8	ND
Pooled	0.331 ± 0.015 108 ± 37		7 ± 1	ND[a]	49 ± 7	16 ± 7	ND
Goat	1.450 ± 0.013	ND	ND	94 ± 21.	ND	ND	81 ± 11

[a] ND, not done.

both A- and B-subfragments existed in human sera, MSPΔ5 could not neutralize the pre-existing protective anti-DT antibodies, and therefore could not prevent the inhibition of the cytotoxicity of UCHT1-CRM9. However, it did block the inhibitory effect of the goat and monkey sera. This prompted the construction of the recombinant immunotoxin, sFv-DT390. The IC_{50} of sFv-DT390 is 4.8×10^{-11} M, 1/16 as potent as UCHT1-CRM9. Like many other single-chain constructs, sFv-DT390 is monovalent as compared to immunotoxins generated with full-length bivalent antibodies. The reduced toxicity in sFv-DT390 could be explained primarily on this affinity difference. Immunotoxins generated with purified F(ab)' fragments of antibodies also show an *in vitro* loss in toxicity (generally a 1.5 log difference) when compared to their counterparts generated with full-length antibodies (22). The toxicity of sFv-DT390 is comparable to that reported for DAB_{486}IL-2 (23). Considering using sFv-DT390 in the clinical treatment, will there be a trade-off in evaluating a potent, but completely neutralized toxin with one which is less potent, but not completely blocked? A conclusive answer can only be derived from clinical trials. However, from the *in vitro* data some advantages of sFv-DT390 can be expected. First, sFv-DT390 is only one-third of the molecular weight of UCHT1-CRM9. Therefore, sFv-DT390 can penetrate into tissue more readily. Second, in an *in vitro* experiment (Table III), the same molar concentration of sFv-DT390 and UCHT1-CRM9 was used for the serum inhibition test, although the former is only 1/16 potent compared to the latter. The pre-existing anti-DT antibodies in human sera could only partially block the toxicity of sFv-DT390 while the effect of UCHT1-CRM9 was completely blocked. Thus, sFv-DT390 could potentially bypass the anti-DT antibodies in *in vivo* situations while UCHT1-CRM9 cannot. Third, sFv-DT390 contains only the variable region of UCHT1, and should have less immunogenicity in human anti-mouse antibody responses than the native murine antibody UCHT1. Finally, the production cost of sFv-DT390 is much lower than that of UCHT1-CRM9. Based on these reasons, it is conceivable that sFv-DT390, or others with similar properties, should be useful in the treatment of T-cell mediated diseases in humans, especially in anti-DT positive individuals and in patients who need repeated treatments. To obtain evidence supporting this assumption, a rhesus monkey analog of sFv-DT390 is currently being constructed in this laboratory, and will be tested in monkey models.

In summary, this report demonstrates that human sera can affect the toxicity and therefore the efficacy of immunotoxins generated with full-length DT mutants. It also indicates that there is a difference in anti-DT antibody repertoire between the humans and non-human primates, suggesting the immunotoxins destined for clinical trials be investigated with the appropriate serum. Furthermore, a truncated DT mutant has been suggested for generation of immunotoxins to bypass the blocking effect of pre-existing anti-DT antibodies in humans.

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